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DOCKET NO. 17282CIP(BOT)
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Steward et al
U.S. Patent: 6,843,998 B1
Serial No.: 09/548,409
Issued: Jan. 18, 2005
Filed: Apr. 13, 2000

For: METHODS AND COMPOSITIONS FOR THE TREATMENT OF PANCREATITIS

Commisioner for Patents
Alexandria, VA 22313-1450

REQUEST FOR
CERTIFICATE OF CORRECTION
UNDER RULE 322 (OFFICE MISTAKE)

Dear Sir:

Please correct the above-identified patent as shown on the accompanying Certificate of Correction Form PTO-1050.

These corrections are requested for the following reasons:

IN THE SPECIFICATION:

'0

Column 1, line 35 (Page 2, line 11); delete "juiced" and insert in place thereof --juice--
Column 10, line 38 (Page 23, line 8); delete "Phe_{189}" and insert in place thereof --Phe_{198}--
Column 17, line 35 (Page 32, line 39); delete "all." and insert in place thereof --all--

Please send the Certificate to:

Allergan, Inc.
Dean G. Stathakis, Ph.D. (T2-7H)
Intellectual Property Dept.
2525 Dupont Drive
Irvine, CA 92612

Respectfully Submitted,

Dean G. Stathakis
Registration No. 54,465

Telephone: 714/246-6521;Telecopier: 714/246-4249

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I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST-CLASS MAIL WITH SUFFICIENT POSTAGE IN AN ENVELOPE ADDRESSED TO THE: CERTIFICATE OF CORRECTION-NON FEE; COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VA 22313-1450 ON 4/15/05 Printed Name of Person Making Deposit: Bonnie Ferguson; Signature of Person Making Deposit: Bonnie Ferguson
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PTO/SB/44 (02-01)
Approved for use through 01/31/2004. OMB 0651-0033

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO: 6,843,998 B1

DATED: Jan. 18, 2005

INVENTORS: Steward et al

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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MAILING ADDRESS OF SENDER: Dean G. Stathakis (T2-7H) 17282CIP(BOT)
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PTO-Form 1050

PTO/SB/44 (02-01)
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METHODS AND COMPOSITIONS FOR THE TREATMENT OF PANCREATITIS

This application is a continuation-in-part of application Ser. No. 09/288,326, filed Apr. 8, 1999.

FIELD OF THE INVENTION

The present invention includes methods and compositions for the treatment of acute pancreatitis. In a preferred embodiment the invention concerns the use of agents to reduce or prevent the secretion of pancreatic digestive enzymes within the pancreas. Such agents are targeted to pancreatic cells, and serve to prevent the exocytotic fusion of vesicles containing these enzymes with the plasma membrane. The invention is also concerned with methods of treating a mammal suffering from pancreatitis through the administration of such agents.

BACKGROUND OF THE INVENTION

Pancreatitis is a serious medical condition involving an inflammation of the pancreas. In acute or chronic pancreatitis the inflammation manifests itself in the release and activation of pancreatic enzymes within the organ itself, leading to autodigestion. In many cases of acute pancreatitis, the condition can lead to death.

In normal mammals, the pancreas, a large gland similar in structure to the salivary gland, is responsible for the production and secretion of digestive enzymes, which digest ingested food, and bicarbonate for the neutralization of the acidic chyme produced in the stomach. The pancreas contains acinar cells, responsible for enzyme production, and ductal cells, which secrete large amounts of sodium bicarbonate solution. The combined secretion product is termed "pancreatic juice"; this liquid flows through the pancreatic duct past the sphincter of Oddi into the duodenum. The secretion of pancreatic juice is stimulated by the presence of chyme in the upper portions of the small intestine, and the precise composition of pancreatic juice appears to be influenced by the types of compounds (carbohydrate, lipid, protein, and/or nucleic acid) in the chyme.

The constituents of pancreatic juice includes proteases (trypsin, chymotrypsin, carboxypolypeptidase), nucleases (RNAse and DNAse), pancreatic amylase, and lipases (pancreatic lipase, cholesterol esterase and phospholipase). Many of these enzymes, including the proteases, are initially synthesized by the acinar cells in an inactive form as zymogens: thus trypsin is synthesized as trypsinogen, chymotrypsin as chymotypsinogen, and carboxypolypeptidase as procarboxypolypeptidase. These enzymes are activated according to a cascade, wherein, in the first step, trypsin is activated through proteolytic cleavage by the enzyme enterokinase. Trypsinogen can also be autoactivated by trypsin; thus one activation has begun, the activation process can proceed rapidly. Trypsin, in turn, activates both chymotypsinogen and procarboxypolypeptidase to form their active protease counterparts.

The enzymes are normally activated only when they enter the intestinal mucosa in order to prevent autodigestion of the pancreas. In order to prevent premature activation, the acinar cells also co-secrete a trypsin inhibitor that normally prevents activation of the proteolytic enzymes within the secretory cells and in the ducts of the pancreas. Inhibition of trypsin activity also prevents activation of the other proteases.

Pancreatitis can occur when an excess amount of trypsin saturates the supply of trypsin inhibitor. This, in turn, can be caused by underproduction of trypsin inhibitor, or the overabundance of trypsin within the cells or ducts of the pancreas. In the latter case, pancreatic trauma or blockage of a duct can lead to localized overabundance of trypsin; under acute conditions large amounts of pancreatic zymogen secretion can pool in the damaged areas of the pancreas. If even a small amount of free trypsin is available activation of all the zymogenic proteases rapidly occurs, and can lead to digestion of the pancreas (acute pancreatitis) and in particularly severe cases to the patient's death.

Pancreatic secretion is normally regulated by both hormonal and nervous mechanisms. When the gastric phase of stomach secretion occurs, parasympathetic nerve impulses are relayed to the pancreas, which initially results in acetylcholine release, followed by secretion of enzymes into the pancreatic acini for temporary storage.

When acid chyme thereafter enters the small intestine, the mucosal cells of the upper intestine release a hormone called secretin. In humans, secretin is a 27 amino acid (3400 Dalton) polypeptide initially produced as the inactive form prosecretin, which is then activated by proteolytic cleavage. Secretin is then absorbed into the blood. Secretin causes the pancreas to secrete large quantities of a fluid containing bicarbonate ion. Secretin does not stimulate the acinar cells, which produce the digestive enzymes. The bicarbonate fluid serves to neutralize the chyme and to provide a slightly alkaline optimal environment for the enzymes.

Another peptide hormone, cholecystokinin (CCK) is released by the mucosal cells in response to the presence of food in the upper intestine. As described in further detail below, human CCK is synthesized as a protoprotein of 115 amino acids. Active CCK forms are quickly taken into the blood through the digestive tract, and normally stimulate the secretion of enzymes by the acinar cells. However, stimulation of the CCK receptor by the CCK analogs cerulein and CCK-octapeptide (CCK-8) appears to lead to a worsening of morbidity and mortality in mammals in whom pancreatitis is induced. See Tani et al., *Pancreas* 5:284–290 (1990).

As indicated above, the digestive enzymes are synthesized as zymogens; proto-enzyme synthesis occurs in the rough endoplasmic reticulum of the acinar cells. The zymogens are then packaged within vesicles having a single lipid bilayer membrane. The zymogens are packed within the vesicles so densely that they appear as quasi-crystalline structures when observed under light microscopy and the zymogen granules are electron-dense when observed under the electron microscope. The vesicles are localized within the cytoplasm of the acinar cells. Secretion of zymogens by the acinar cells occurs through vesicle docking and subsequent fusion with the plasma membrane, resulting in the liberation of the contents into the extracellular milieu.

Nerve cells appear to secrete neurotransmitters and other intercellular signaling factors through a mechanism of membrane fusion that is shared with other cell types, see e.g., Rizo & Sudhof, *Nature Struct. Biol.* 5:839–842 (October 1998), hereby incorporated by reference herein, including the pancreatic acinar cells.

Although the Applicants do not wish to be bound by theory, it is believed that a vesicle first contacts the intracellular surface of the cellular membrane in a reaction called docking. Following the docking step the membrane fuses with and becomes part of the plasma membrane through a series of steps that currently remain relatively uncharacterized, but which clearly involve certain vesicle and membrane-associated proteins, as has been illustrated using neural models.

digestive enzymes, which digest ingested food, and bicarbonate for the neutralization of the acidic chyme produced in the stomach. The pancreas contains acinar cells, responsible for enzyme production, and ductal cells, which secrete large amounts of sodium bicarbonate solution. The combined secretion product is termed "pancreatic juice"; this liquid flows through the pancreatic duct past the sphincter of Oddi into the duodenum. The secretion of pancreatic juice is stimulated by the presence of chyme in the upper portions of the small intestine, and the precise composition of pancreatic juice appears to be influenced by the types of compounds (carbohydrate, lipid, protein, and/or nucleic acid) in the chyme.

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done either by incorporation of commercially available Fmoc-Tyr(OSO_3^-)-OH into the peptide chain at the 7th amino acid position prior to cleavage of the synthetic peptide from the solid support hereby incorporated by reference herein), or by standard peptide synthesis using tyrosine at position 7, followed by a sulfation reaction of the peptide resulting in tyrosine sulfate at the 7 position. See e.g., Koeller, K. M., *J. Am. Chem. Soc.* 122:742–743 (2000). The synthetic peptide is constructed with a cysteine (or serine or threonine) residue at the amino terminus.

It will be understood that one can use either hydroxyl-containing amino acids or cysteine as the amino terminal residue of the intein and the synthetic peptide, and either thiopheol, phenol or another nucleophile capable of creating a reactive ester or thioester linkage in accordance with the expressed protein ligation methods described herein. However, thiol-containing amino acid residues and thipheonol or another sulfur-containing nucleophile are preferred.

Thus, according to one embodiment of the expressed protein ligation method, the fusion protein is immobilized following expression by incubation under selective binding conditions with a surface to which the binding partner of the carboxyl terminal has been joined (e.g., where the binding moiety is CBP, the surface may be a resin to which chitin is conjugated). The immobilized fusion protein is then permitted to react in a transthioesterification reaction with a S- or O-containing reagent (such as thiophenol or phenol) and the synthetic modified peptide described above. In this step, the intein which is joined to the carboxyl terminus of the therapeutic polypeptide is cleaved at the thioester (or ester) linkage, thus liberating the protein from the surface to which it was bound. The intein may be transiently replaced with the thiophenol group, and the resulting thioester is then itself attacked by the cysteine (or serine or threonine) residue of the synthetic peptide; this reaction is then spontaneously followed by a shift of the carbonyl bond from S (or O) to the N terminal nitrogen of the synthetic peptide, to form a peptide bond. The resultant therapeutic polypeptide thus comprises a threapeutic domain, a translocation domain, and a binding domain comprising a CCK sequence modified to contain the naturally occuring post-translational modifications.

As intended herein, the term "extein" refers to a portion of a chimeric polypeptide that borders one or more intein, and is subsequently ligated to either another extein or a synthetic polypeptide in the EPL reaction referred to herein.

As intended herein, the term "intein" refers to a portion of a chimeric polypeptide containing an N-terminal cysteine, serine, or threonine which is excised from said polypeptide during the EPL reaction referred to herein.

Of course, the Applicants contemplate that this method of producing a CCK-containing therapeutic polypeptide is exemplary only, and that variations and modification of the above-described method will be well within the ability and knowledge of those of ordinary skill in the art in light of the present patent application.

While it will be understood that the applicants do not wish to be bound by theory, the following findings may assist an understanding the nature of the interaction between CCK and the CCK receptors, and thus between the CCK receptor binding element of an embodiment of the present invention and its CCK receptor target.

In pancreatic acinar cells the CCK A receptor undergoes internalization to intracellular sites within minutes after agonist exposure. Pohl et al., *J. Biol. Chem.* 272: 18179–18184 (1997), hereby incorporated by reference herein. The CCK B receptor has also shown the same ligand-dependant internalization response in transfected NIH 3T3 cells. In the CCK B receptor, but not the CCK A receptor, the endocytotic feature of the receptor been shown to be profoundly decreased by the deletion of the C terminal 44 amino acids of the receptor chain, corresponding in both receptors to an cytoplasmic portion of the receptor chain.

Recent studies of the interaction between the CCK A receptor and CCK have shown that the primary receptor sequence region containing amino acid residues 38 through 42 is involved in the binding of CCK. Residues Trp_{39} and Gln_{40} appear to be essential for the binding of a synthetic CCK C-terminal nonapeptide (in which the methionine residues located at residue 3 and 6 from the C-terminus are substituted by norleucine and threonine respectively) to the receptor. Kennedy et al., supra. These residues do not appear to be essential for the binding of CCK analogs JMV 180 (corresponding the synthetic C-terminal heptapeptide of CCK in which the phenylalanylamide residue is substituted by a phenylethyl ester and the threonine is substituted with norleucine), and JMV 179 (in which the phenylalanylamide residue and the L-tryptophan residues of the synthetic CCK nonapeptide are substituted by a phenylethyl ester and D-tryptophan, respectively and the threonine is substituted with norleucine). Id.

These and similar studies have shed light on the structure of the CCK A receptor active site. Based on receptor binding experiments, a current structural model indicates that CCK residues Trp_{30} and Met_{31} (located at positions 4 and 3, respectively, from the C terminus of mature CCK-8) reside in a hydrophobic pocket formed by receptor residues Leu_{348}, Pro_{352}, Ile_{353} and Ile_{356}. CCK residue Asp_{32} (located at amino acid position 2 measured from the C terminus of CCK-8) seems to be involved in an ionic interaction with receptor residue Lys_{115}. CCK Tyr-sulfate$_{27}$ (the CCK-8 residue 7 amino acids from C terminus) appears involved in an ionic interaction with receptor residue Lys_{105} and a stacking interaction with receptor residue Phe_{189}. Ji, et al., 272 *J. Biol. Chem.* 24393–24401 (1997).

Such structural models provide detailed guidance to the person of ordinary skill in the art as to the construction of a variety of binding elements able to retain the binding characteristics of biologically active CCK peptides for the CCK-A receptor, for example, as, for example, by site directed mutagenesis of a clostridial neurotoxin heavy chain. Similarly, models deduced using similar methodologies have been proposed for the CCK B receptor, see e.g., Jagerschmidt, A. et al., *Mol. Pharmacol.* 48:783–789 (1995), and can be used as a basis for the construction of binding elements that retain binding characteristics similar to the CCK B receptor.

It will be appreciated that the CCK-B receptor is known to exist on the surface of neurons associated with the certal nervious system. In one alternative embodiment of the present invention the therapeutic polypeptide may be directed (for example, by intrathecal application) to these neurons rather than to the pancreas); in such a case, the binding element may comprise a CCK containing the C terminal amidation only. Such a binding element may be constructed using the expressed protein ligation (EPL) methods described above. Indeed, EPL methods may be used to introduce and desired or required modifications to the therapeutic element, the translocation element, and/or the binding element of the claimed therapeutic polypeptide.

Additionally, the binding element may comprise a variable region of an antibody which will bind the CCK-A or CCK-B receptor.

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gtaatgaagt caaaaaatga tcaaggaata acaaataaat gcaaaatgaa tttacaagat
aataatggga atgatatagg ctttatagga tttcatcagt ttaataatat agctaaacta
gtagcaagta attggtataa tagacaaata gaaagatcta gtaggacttt gggttgctca
tgggaattta ttcctgtaga tgatggatgg ggagaaaggc cactgtaatt aatctcaaac
tacatgagtc tgtcaagaat tttctgtaaa catccataaa aattttaaaa ttaatatgtt
taagaataac tagatatgag tattgtttga actgcccctg tcaagtagac aggtaaaaaa
ataaaaatta agatactatg gtctgatttc gatattctat cggagtcaga ccttttaact
tttcttgtat ccttttttgta ttgtaaaact ctatgtattc atcaattgca agttccaatt
agtcaaaatt atgaaacttt ctaagataat acatttctga ttttataatt tcccaaaatc
cttccatagg accattatca atacatctac caactcgaga catactttga gttgcgccta
tctcattaag tttattcttg aaagatttac ttgtatattg aaaaccgcta tcactgtgaa
aaagtggact agcatcagga ttggaggtaa ctgctttatc aaaggtttca aagacaagga
cgttgttatt tgattttcca agtacatagg aaataatgct attatcatgc aaatcaagta
tttcactcaa gtacgccttt gtttcgtctg ttaac

Of course, three distinct domains analogous to those described above for BoNT/A exist for all the BoNT subtypes as well as for TeNT neurotoxin; an alignment of the amino acid sequences of these holotoxins will reveal the sequence coordinates for these other neurotoxin species. Additionally, while sequence information is given above for BoNT/A, the amino acid sequences of all BoNT species and tetanus toxin TeNT are known and can easily be obtained from, for example, the NCBI Gen-Bank Web site: www.ncbi.nlm.nih.gov. The Clostrdial neurotoxin nucleotide and amino acid sequences disclosed at this site are expressly incorporated by reference herein.

-continued

gtagaaaaaa tactaagtgc attagaaata cctgatgtag gaaatctaag tcaagtagta

gtaatgaagt caaaaaatga tcaaggaata acaaataaat gcaaaatgaa tttacaagat

aataatggga atgatatagg ctttatagga tttcatcagt ttaataatat agctaaacta

gtagcaagta attggtataa tagacaaata gaaagatcta gtaggacttt gggttgctca

tgggaattta ttcctgtaga tgatggatgg ggagaaaggc cactgtaatt aatctcaaac

tacatgagtc tgtcaagaat tttctgtaaa catccataaa aattttaaaa ttaatatgtt

taagaataac tagatatgag tattgtttga actgcccctg tcaagtagac aggtaaaaaa

ataaaaatta agatactatg gtctgatttc gatattctat cggagtcaga ccttttaact

tttcttgtat ccttttttgta ttgtaaaact ctatgtattc atcaattgca agttccaatt

agtcaaaatt atgaaacttt ctaagataat acatttctga ttttataatt tcccaaaatc

cttccatagg accattatca atacatctac caactcgaga catactttga gttgcgccta

tctcattaag tttattcttg aaagatttac ttgtatattg aaaaccgcta tcactgtgaa

aaagtggact agcatcagga ttggaggtaa ctgctttatc aaaggtttca aagacaagga

cgttgttatt tgattttcca agtacatagg aaataatgct attatcatgc aaatcaagta

tttcactcaa gtacgccttt gtttcgtctg ttaac

Of course, three distinct domains analogous to those described above for BoNT/A exist for all the BoNT subtypes as well as for TeNT neurotoxin; an alignment of the amino acid sequences of these holotoxins will reveal the sequence coordinates for these other neurotoxin species. Additionally, while sequence information is given above for BoNT/A, the amino acid sequences of all BoNT species and tetanus toxin TeNT are known and can easily be obtained from, for example, the NCBI Gen-Bank Web site: world wide web ncbi.nlm.nih.gov. The Clostrdial neurotoxin nucleotide and amino acid sequences disclosed at this site are expressly incorporated by reference herein.

Preferably, the translocation element and the binding element of the compositions of the present invention are separated by a spacer moiety that facilitates the binding element's binding to the desired cell surface receptor. Such a spacer may comprise, for example, a portion of the BoNT H_C sequence (so long as the portion does not retain the ability to bind to the BoNT or TeNT binding site of motor neurons or sensory afferent neurons), another sequence of amino acids, or a hydrocarbon moiety. The spacer moiety may also comprise a proline, serine, threonine and/or cysteine-rich amino acid sequence similar or identical to a human immunoglobulin hinge region. In a preferred embodiment, the spacer region comprises the amino acid sequence of an immunoglobulin γl hinge region; such a sequence has the sequence (from N terminus to C terminus):

EPKSCDKTHTCPPCP (SEQ ID NO:11)

It will be understood that none of the examples or embodiments described herein are to be construed as limiting the scope of the invention, which is defined solely by the claims that conclude this specification.

EXAMPLE 1

An agent for the treatment of acute pancreatitis is constructed as follows.

A culture of *Clostridium botulinum* is permitted to grown to confluence. The cells are then lysed and total RNA is extracted according to conventional methods and in the presence of an RNAse inhibitor. The RNA preparation is then passed over a oligo(dT) cellulose column, the polyadenylated messenger RNA is permitted to bind, and the column is washed with 5–10 column volumes of 20 mM Tris pH 7.6, 0.5 M NaCl, 1 mM EDTA (ethylenediamine tetraacetic acid), 0.1% (w/v)SDS (sodium dodecyl sulfate). Polyadenylated RNA is then eluted with 2–3 column volumes of STE (10 mM Tris (pH 7.6), 1 mM EDTA, 0.05% (w/v) SDS). The pooled mRNA is then precipitated in 2 volumes of ice cold ethanol, pelleted in a centrifuge at 10,000×g for 15 minutes, then redissolved in a small volume of STE.

The BoNT/A mRNA is used as a template for DNA synthesis using Moloney murine leukemia virus reverse transcriptase (MMLV-RT), then the L chain and then H_N chain of the neurotoxin is amplified from the cDNA by the polymerase chain reaction (PCR) using appropriate oligonucleotide primers whose sequences are designed based on the BoNT/A neurotoxin cDNA sequence of SEQ ID NO: 9. These procedures are performed using the standard techniques of molecular biology as detailed in, for example, Sambrook et al., already incorporated by reference herein. The primer defining the beginning of the coding region (5' side of the L chain fragment) is given a StuI site. The PCR primer defining the 3' end of the H_N-encoding domain has the following features (from 3' to 5'): a 5' region sufficiently complementary to the 3' end of the HN-encoding domain to anneal thereto under amplification conditions, a nucleotide sequence encoding the human immunoglobulin hinge region γ_1 (SEQ ID NO:11), a nucleotide sequence encoding the human CCK-8 octapeptide (SEQ ID NO:6), and a unique restriction endonuclease cleavage site.

The PCR product (termed BoNT/A$^{L\text{-}HN\text{-}\gamma\text{-}CCK}$) is purified by agarose gel electrophoresis, and cloned into a pBluescript II SK vector. The resulting plasmid is used to transform competent *E. coli* cells, and a preparation of the resulting plasmid is made. The BoNT/A$^{L\text{-}HN\text{-}\gamma\text{-}CCK}$ fragment is excised from the pBluescript vector and cloned into a mammalian expression vector immediately downstream of a strong promoter. The resulting vector is used to transfect a culture of the appropriate host cell, which is then grown to